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Sun exposure as a risk factor for precipitating vision loss for individuals with LHON mitochondrial variants

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Abstract

Vision loss in Leber hereditary optic neuropathy (LHON, OMIM# 535000) is caused by pathogenic LHON associated mitochondrial DNA (mtDNA) variants that are often considered to be triggered by a second environmental stress (genotype-environmental double hit). A few studies have demonstrated associations between the onset of LHON vision loss and specific environmental stresses such as tobacco or alcohol use. Other plausible triggers, such as UV exposure through sunlight, has not yet been studied. The purpose of this study was to survey LHON patients' lifestyle and habits to investigate the risk of light exposure on this population, including the correlation of the time of year vision-loss was triggered. We collected information on their daily smoking habits, alcohol use, and sun exposure for months before and during the time of vision loss. We did not find a strong correlation between sun exposure and onset of vision loss, though we cannot rule out a contribution in precipitating the LHON phenotype since individuals who lost their vision in adulthood reported longer sun exposure, and more total exposures, than individuals who lost their vision in youth. This study contributes to the growing body of literature regarding environmental influences of LHON blindness, and we suggest that UV exposure still requires further investigation to understand what, if any, role it may play.

Key words

Exposure, Mitochondria, LHON, Light Damage, Environmental Triggers

Introduction

Leber hereditary optic neuropathy (LHON) is one of the most common mitochondrial disorders. The lifetime risk for visual failure in individuals with a LHON-causing mitochondrial DNA is higher in men as compared to women (Van Senu 1963; Mackey 1994a; E. K. Nikoskelainen et al. 1987; Mackey 1994b). Ninety-five percent of LHON carriers will have one of three mitochondrial missense variants in genes encoding subunits of NADH dehydrogenases ND4 (mt.11778G>A), ND6 (mt.14484T>C), or ND1 (mt.3460G>A) (Brown, Voljavec, Lott, MacDonald, & Wallace, 1992; Howell, Kubacka, Xu, & McCullough, 1991; Wallace et al., 1988). Mitochondrial NADH dehydrogenase (ND) is part of Complex I of the electron transport chain, important for the production of a proton gradient and the reduction of ubiquinone to ubiquinol. The redox reaction at complex I is not 100% efficient and some of the electrons are lost as reactive oxygen species (ROS) (Alberts et al., 2002). Pathogenic LHON variants in complex I lead to an increase in the generation of ROS (Hofhaus & Attardi, 1993; Yen, Lee, Liu, & Wei, 1996). Furthermore, the optic nerve head of the eye is extremely energy demanding as it is a meeting point for nerve axons and is particularly vulnerable to energy depletion (Carelli, Ross-Cisneros, & Sadun, 2002).

The pathogenicity of LHON variants are multifactorial; both genetic and environmental factors contribute to the phenotype (L. Giordano et al., 2015; Smith, Cooper, Govan, Harding, & Schapira, 1993). Thus, the identification of trends in patient data are complicated by inherent genetic factors that modify the severity of the LHON phenotype. Harmful environmental factors previously investigated

include smoking (Kirkman et al., 2009), and alcohol consumption (Sadun et al., 2004). Though less studied, there are also case reports of trauma (Apinyawasisuk, Chan, & Arnold, 2016), antiretroviral therapy and HIV (Shaikh, Ta, Basham, & Mansour, 2001), anemia (Goyal, Riordan-Eva, & Coakes, 2004), cyanide exposure (Berninger, von Meyer, Siess, Schon, & Goebel, 1989), fumes from a tire fire (Sanchez, Smith, Carelli, Sadun, & Keltner, 2006), solvents such as n-hexane (Carelli et al., 2007), ethambutol medication (Ikeda, Ikeda, Ikeda, Kawakami, & Mimura, 2006), and carbon monoxide intoxication (Hwang & Park, 1996) influencing the LHON phenotype. Many of the aforementioned triggers likely exacerbate LHON through oxidative stress and/or direct mitochondrial toxicity. Visual recovery reported is highest for individuals with the mt.14484T>C variant, however has been reported for all three LHON variants, and may be due to an alleviation of mitochondrial stress (Mashima, Kigasawa, Wakakura, & Oguchi, 2000).

Light has been shown to be a source of oxidative stress for both the mitochondria and the mitochondria-rich retinal ganglion cells (Osborne, Lascaratos, Bron, Chidlow, & Wood, 2006; Osborne, Li, Ji, Mortiboys, & Jackson, 2008). Isolated mitochondria exposed to light in the range of 400-760 nm had reduced mitochondrial function, including redox reactions and mitochondrial dehydrogenase activity, which was a function of light intensity (Osborne, Lascaratos, Bron, Chidlow, & Wood, 2006). Furthermore, studies suggest that light in the range of 400-760 nm causes damage to retinal ganglion cells in tissue culture when exposed to 1000 Lux of light for 24 and 48 hours (1,000 Lux is

equivalent to exposure on an overcast day, as compared to bright sunlight, which is about 111,000 Lux) (Osborne, Li, Ji, Mortiboys, & Jackson, 2008). The light exposure on these tissue culture cells also resulted in a significant amount of ROS species generation (Osborne, Li, Ji, Mortiboys, & Jackson, 2008). Since light can induce retinal damage leading to the generation of reactive oxidative species and apoptosis of retinal ganglion cell loss, our *a priori* hypothesis was that higher amounts of light exposure and/or acute light exposure around the time of vision loss could contribute to triggering the pathophysiology and lead to visual loss in LHON patients.

Methods

Recruitment of participants with LHON

Participants were individuals with LHON who had experienced vision loss and were invited to take part in a survey designed to collect information on smoking habits, alcohol use, and sun exposure for months before and during the time of vision loss. Participants were recruited internationally by advertisements in the monthly LHON Support Group newsletter (<https://www.lhon.org>) or through postings on the LHON Support Group Facebook page (Leber's Hereditary Optic Neuropathy (LHON) (BLIND), <https://www.facebook.com/groups/29805437752>). The survey was provided in English only. Participants were also given the option to make an appointment with the first author to take the survey over the phone.

Survey and protocol for the study entitled LE-LHON was approved by the McMaster Research Ethics Board (Project ID: 4852) and the Sarah Lawrence Internal Review Board. All procedures followed were in accordance with the ethical

standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000 (5). Informed consent was obtained from all patients included in the study. Respondents could only complete the survey after checking a box indicating informed consent to participate. Our cohort was representative of the LHON population, in which vision loss tends to occur between the ages of 15 to 35 years of age and the m.11778G>A variant was the most common LHON variant observed.

Survey Instrument and data collection

The survey was created and administered through McMaster University Limesurvey (Schmitz, 2012). The survey contained four sections: Preamble and Consent; Personal Demographics; LHON Vision Information; and Possible Exposures. Qualitative and quantitative questions were aimed at investigating possible exposures that may have precipitated vision loss, including smoking, drinking, chemicals and sunlight (**Sup. Table 1**). Data on visual recovery was also collected.

Data analysis

Graphs were generated and statistical analysis was performed on GraphPad Prism 5 and GraphPad Prism 8 (Swift, 1997). World map was downloaded from: <https://www.mytravelmap.xyz/?1>. Yearly average sunlight was adapted from: <http://earth.rice.edu/mtpe/geo/geosphere/hot/energyfuture/sunlight.html> (Earth Forum, Houston Museum of Natural Science; Data from the World Resources

Institute). Hours of sunlight on the day of vision loss was calculated using <https://www.timeanddate.com/sun/usa>, and only if the participant provided a location and a date were they included in this particular analysis.

Results

Our survey was completed by 47 participants, most identified as male (68%), white (85%), and non-smokers (68%, **Fig. 1A**). The most common mtDNA genetic variant was m.11778G>A (63.83%), although there were many individuals regarded as having LHON and did not state or report knowing their pathogenic gene changes (**Fig. 1B**). The range of vision loss in our cohort was 8-69 years old (**Fig. 1C**). In addition, median age of vision loss for individuals with the m.11778G>A variant was younger (25 years old), than the other variants. Specifically, median was 28 years old for people with the m.3460G>A variant, 30.5 years old for people with the m.14484T>C variant and 34 years old for individuals with unknown genetic variants (Data not shown). Males in the study had an earlier onset of vision loss (**Fig. 1D**). Median age of onset for males was 22.5 years old, whereas the median for females was 34 years old. Visual recovery was reported in 23% of participants with the m.11778G>A variant, 33% in participants with m.3460G>A, and 50% in participants with the m.14484T>C variant (**Sup. Table 2**).

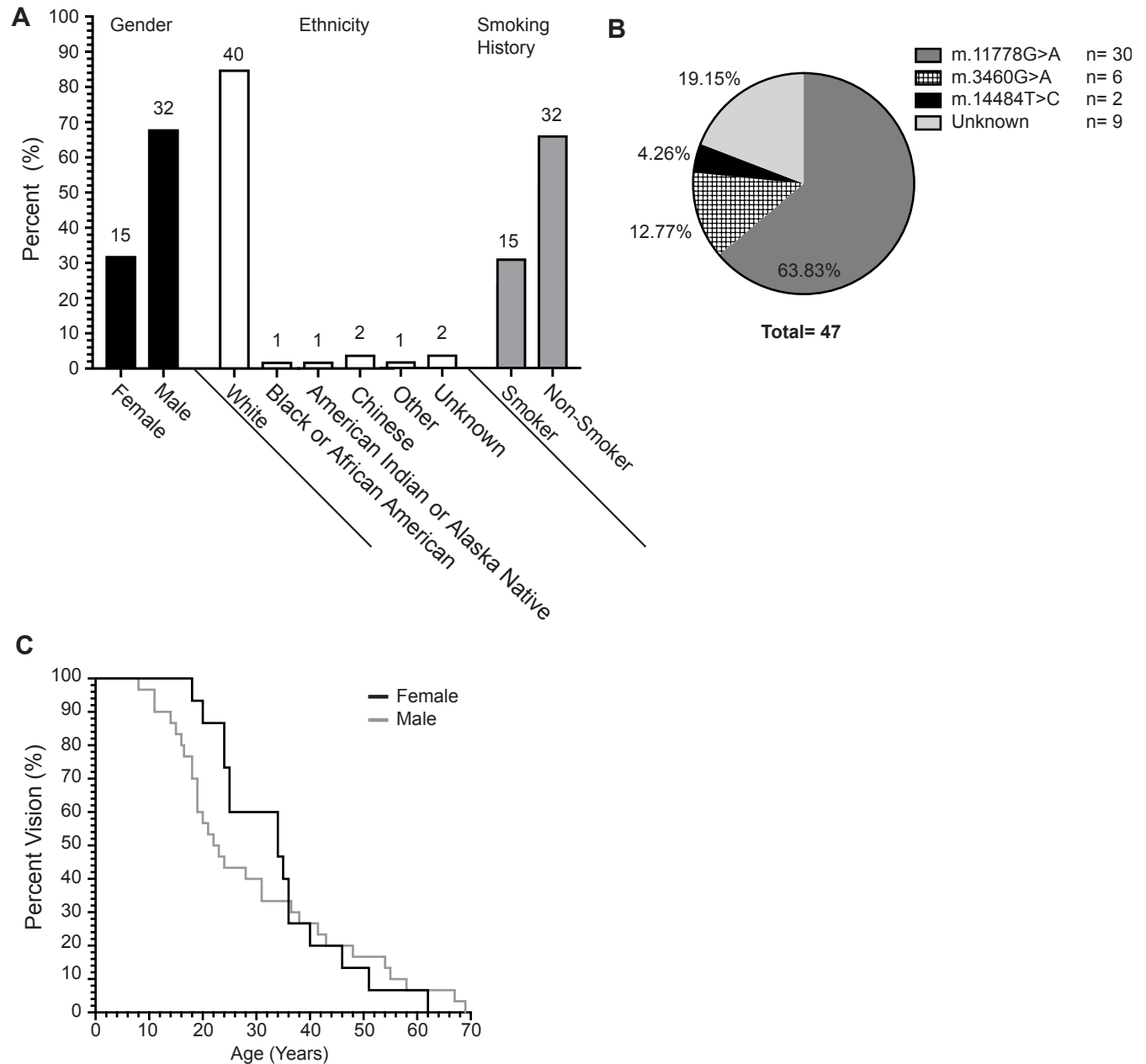


Figure 1. Patient demographics. **(A)** Number of female and male participants (black bars), ethnicities of participants (white bars), and smoking status of participants (grey bars). **(B)** mtDNA variant distribution for the participants in our study. **(C)** Rate of vision loss in our patient population, females (black) and males (grey).

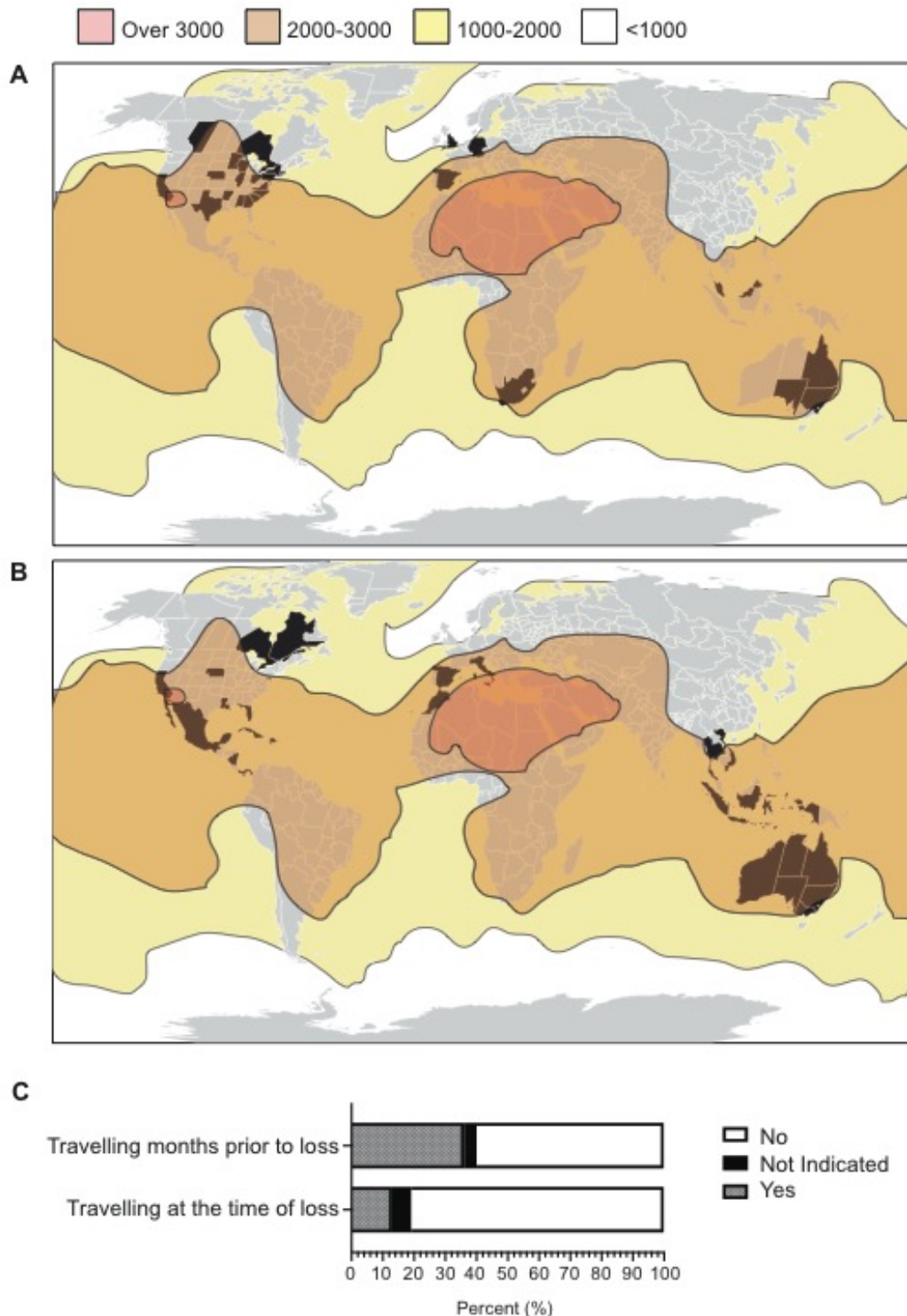


Figure 2. Most participants lived and traveled to areas with moderate-high amount of sunlight during or a few months prior to their vision loss. Areas where participants lived at the time of loss (**A**) or traveled to in the months leading up to the loss (**B**) is shaded in black. Global zones of average yearly total numbers of hours in bright sunlight were shaded with different colors; over

3000 hours is the highest (light red), 2000-3000 hours (light orange), 1000-2000 hours (yellow) and less than 1000 hours (white). **(C)** Total participants travelling at the time of vision loss and prior to vision loss.

We found that most participants reported living in regions with moderate-high quantity of yearly hours of bright sunlight (**Fig. 2A**). Similarly, most traveled in the months prior to a moderate-high zone as well (**Fig. 2B**). Six participants (13%) were travelling at the time they experienced vision loss (**Fig. 2C**), but all travel was reported to occur within the same zone. Seventeen participants (37%) travelled in the months prior to the loss (**Fig. 2C**). Of these, two traveled to zones with higher average yearly total number of hours in bright sunlight. One travelled from England (average 1000-2000 hours) to Spain (average 2000-3000), and the other from Ontario, Canada (average 1000-2000 hours) to Sicily (average over 3000 hours) (**Fig 2A, 2B**). Anecdotally, other participants reported changes in their vision after travel. Travelling to locations with more environmental sunlight might be a risk factor for individuals with LHON pathogenic variants. One participant stated, “2 years ago my vision worsened again after a tourist trip in Portugal (very sunny at that moment). I lost depth sight at that moment.”

In order to assess the possibility of sunlight triggering vision loss, we normalized for location, since different hemispheres experience different seasons. Historical hours of sunlight at the time of vision loss for the right eye and left eye slightly differ in progression (slope for right eye= 0.019, slope for left eye= -0.009), however did not change with age (**Fig. 3A**). Our data indicated that people at any age were not more or less likely to lose their vision in longer hours of light exposure (**Fig. 3A**).

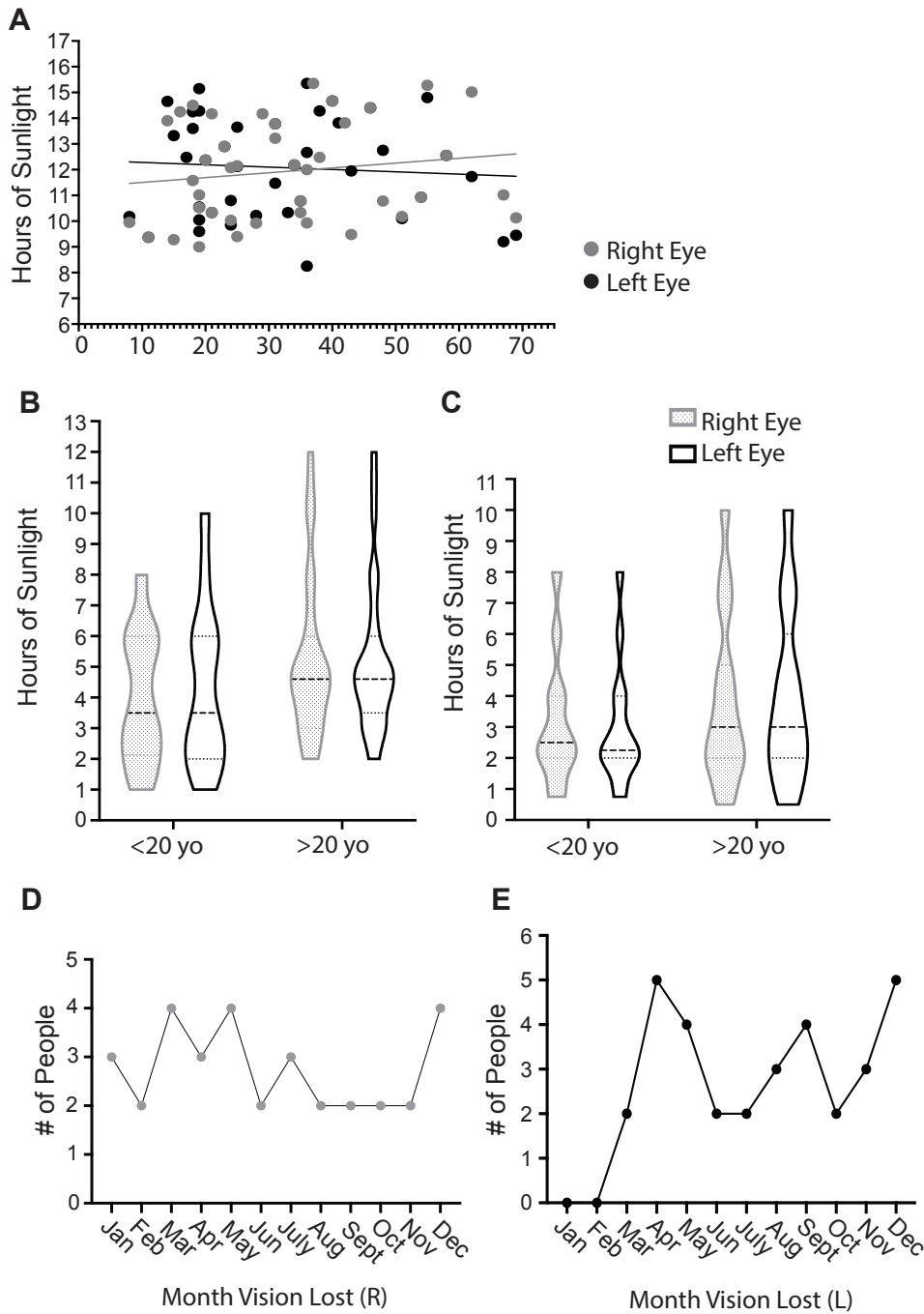


Figure 3. Sun exposure and vision loss in our cohort. **(A)** Hours of sunlight on the day vision was lost in the right eye (grey dots) and left eye (black dots). Line of best fit was calculated through non-linear regression. **(B)** Average daily hours of sunlight during youth for individuals who lost their vision under 20 years old (<20 y) and over 20 years old (>20 y). **(C)** Average hours of sunlight during the months prior to vision loss for individuals under 20 years old (<20 y) and over 20 years old

(>20 y). Months when vision was lost for individuals living in North America (international participants excluded from this graph to normalize for seasons), right eye (**D**) and left eye (**E**).

Next, we checked whether there was a correlation between average daily hours of sunlight reported during youth and subsequent visual loss. Individuals who lost their vision over 20 years of age (>20 y) tended to report longer hours outside during their youth than individuals who lost their vision before the age of 20 (<20 y, **Fig. 3B**), although this difference was not statistically significant (P-value right eye 0.2187 and left eye 0.25014). Average daily hours of sunlight reported for the months prior to vision loss was also higher for the group who lost their vision over the age of 20 years old (**Fig. 3C**), but this too was not statistically significant (P-value for right eye 0.10935 and left eye 0.12507). Finally, normalizing for the individuals exposed to similar seasons (North America), vision loss occurred in almost any month of the year for the right eye and left eye (**Fig. 3D, 3E**). Notably, the months of December, March, April and May, tended to be the most common months that people reported they lost their vision for the right and left eye (**Fig. 3D, 3E**).

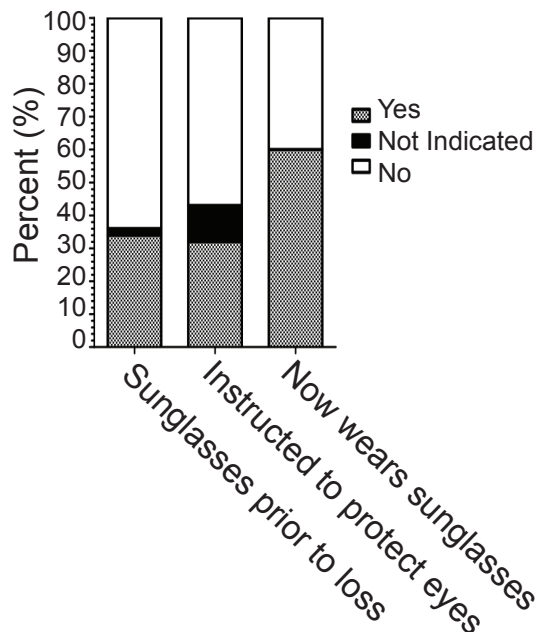


Figure 4. More participants wear sunglasses post vision loss. Participants who wore sunglasses regularly prior to vision loss (column 1), participants told by a medical professional to wear sunglasses prior to vision loss (column 2) and currently wear sunglasses post vision loss (column 3).

The habits surrounding sunglass use changed after the participant lost their vision (**Fig. 4**). Sixteen participants were wearing sunglasses routinely prior to loss (34%), and now 28 wear sunglasses when they are outside (60%, **Fig. 4**). Only 15 were ever told by anyone (medical professional or otherwise) to wear sunglasses (32%), suggesting that it is not common place to consider this a contributing factor to the condition.

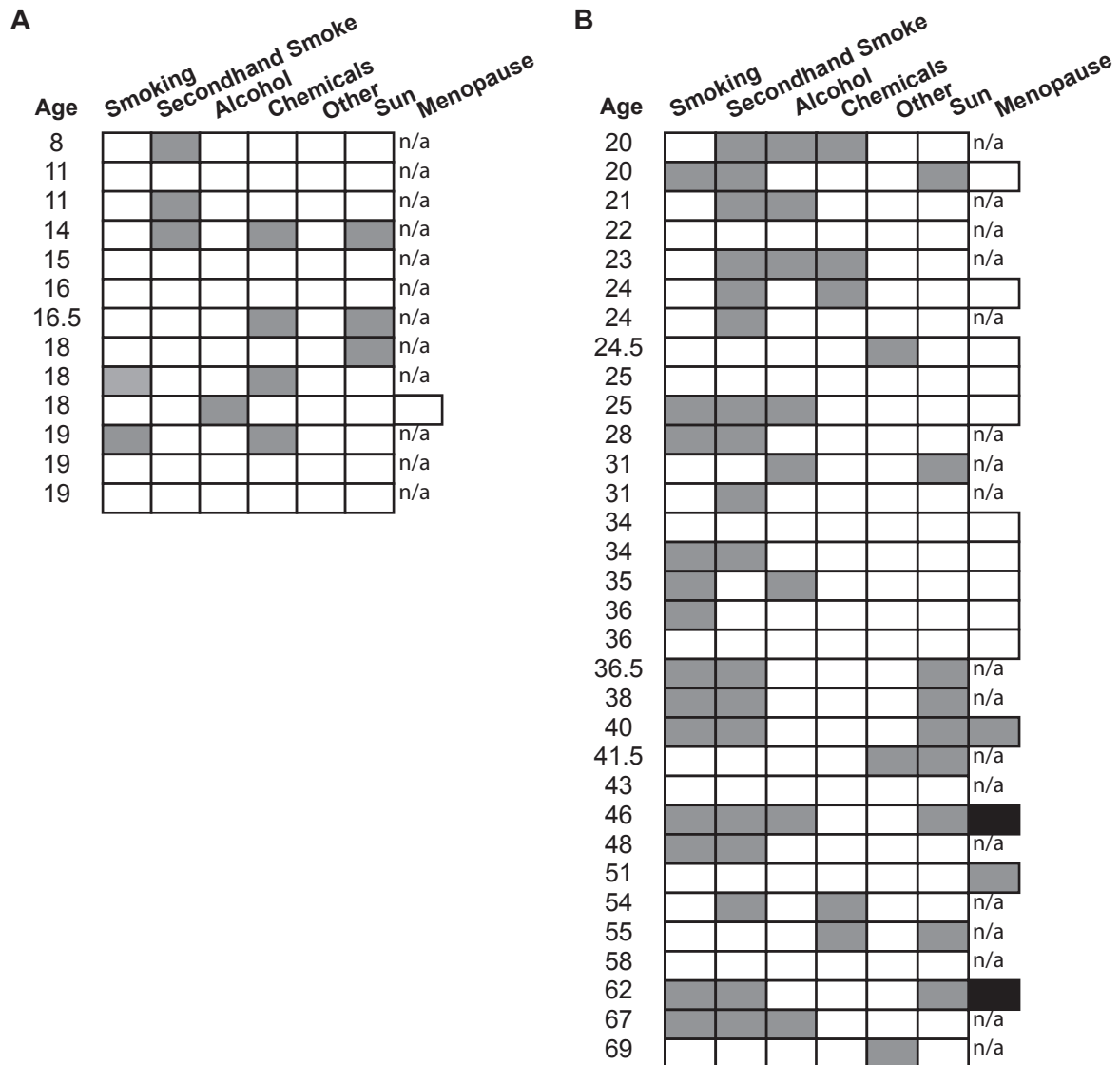


Figure 5. Reported various environmental exposures at the time of vision loss. Shaded grey boxes indicate that the participant responded yes to the assessed exposures. Average age of loss for both right and left eye, oriented from youngest (<20 y, **A**), to oldest (>20 y, **B**). At the time of vision loss; they were a smoker (first column), exposed to secondhand smoke on a regular basis (second column), more than 20 alcoholic beverages per week on average or reported regular binge drinking (third column), exposed to toxic chemicals (fourth column), other reported exposure (fifth column) and more than 13 hours of sunlight (sixth column), and started or completed menopause (last column, n/a if the participant was male). Individuals who had hormone replacement therapy for menopause are indicated in black since some studies suggest receiving the hormone may be protective.

Our survey also collected data on a wide range environmental exposures. Since a LHON mtDNA variant is necessary but not sufficient on its own to trigger the retinal ganglion cell loss, disease conversion is believed to occur through an assortment of environmental factors. Factors considered were exposures based on studies and case reports in the literature, including being a smoker (Kirkman et al., 2009), exposure to secondhand smoke on a regular basis, consumption of an average of more than 20 alcoholic beverages per week or reported regular binge drinking (Sadun et al., 2004), starting or completing menopause (Fantini, Asanad, Karanjia, & Sadun, 2018), exposed to toxic chemicals (specifically mercury, turpentine, DTT or MTBE reported in our cohort) (Carelli et al., 2007; Hwang & Park, 1996), other reported exposures, and more than 13 hours of sunlight at the time of vision loss. Participants over 20 years old on average had two times the number of exposures as individuals under 20 years of age (**Fig. 5A, 5B**). Also, male participants were more susceptible to lose their vision under the age of 20 than female participants (**Fig. 5A, 5B**).

Though not a quantifiable exposure, several participants reported they felt stress was a contributing factor. One participant noted, "In the year and a half leading up to becoming LHON affected I was working very long hours in a very stressful job, and as a result was not sleeping or eating well. I believe that the stress, poor nutrition and sleep were my triggers." Another individual stated, "The loss of vision in my right eye occurred after I gave birth to my son. Since then I have had a hysterectomy so have had changes in hormones, but not officially went

through menopause because one ovary was left.” Finally, for another participant “The heart surgery caused the most vision loss.”

Discussion

Our data series investigates self-reported sunlight exposures and several other possible environmental triggers for precipitating vision loss in a LHON cohort. This is the first-time sunlight has been considered as a risk factor for vision loss in individuals with LHON, although it has long been hypothesized as a contributor for other ocular conditions such as age-related macular degeneration (Zhou, Zhang, Yu, & Xie, 2018) due to *in vitro* studies of blue light induced mitochondrial death (del Olmo-Aguado, Núñez-Álvarez, & Osborne, 2016; Osborne et al., 2008). Current evidence suggests that it is the blue/violet region of the spectrum that causes damage to the retinal ganglion cells (Shang, Wang, Sliney, Yang, & Lee, 2017). However, it is postulated that light does not have a detrimental effect on healthy ganglion cells, because the functioning mitochondria is able to quench the ROS (Osborne et al., 2006).

In this study, participants who experienced vision loss at a younger age reported lower hours of sun exposure than participants who lost vision at an older age (**Fig. 3B, 3C**). Interestingly, studies suggest there is greater UV transmittance to the retina in infancy and childhood than in adulthood (Sanford et al., 1996; Werner & Steele, 1988), suggesting that potentially less light would be required to result in damaging effects in a younger population. In addition, UV absorption by the ocular lens increases with age resulting in less insult reaching the retinal epithelia (Dillon & Atherton, 1990; Werner & Steele, 1988). We cannot discount

that even though younger individuals were exposed to less light on average, this exposure may have been more damaging to a compromised ocular system.

Results from this study suggest that most participants with LHON lived and travelled to areas with moderate-high amount of average yearly sunlight at the time or in the few months prior to their vision loss (**Fig. 2**) Though this study did not identify any statistically significant correlations between vision loss and sunlight exposure, several participants reported anecdotal evidence of vision loss during a time of increased sun exposure (e.g. Trip to sunny location). Sun exposure may have been underestimated since we did not survey participants about specific details of trips or activities that occurred prior to vision loss (e.g. A ski trip, visiting a summer home, or other outdoor activities). Months with the highest likelihood of vision loss for our cohort was April, May and December (**Fig. 3D, 3E**). Vision loss observed in December possibly correlates with skiing or travel, and April with traveling for spring break. Furthermore, travelling itself may be considered a mitochondrial stressor. Altered circadian rhythms may negatively affect mitochondria (Reviewed: Sardon Puig, Valera-Alberni, Cantó, & Pillon, 2018), thus travelling should be quantified and explored as a possible contributor of oxidative stress.

Future follow-up studies, possibly with a larger sample size, may be required to further investigate the timing and surroundings individuals were exposed to around the time of vision loss. This current study may also be limited by participants' recall bias, as their vision loss may have occurred many years in the past.

Quantification of sunlight exposure is also challenged by multiple other variables in an individual's day-to-day environment. Geometrical exposures, such as measuring sun angle, ground surface albedo, and light blocking apparel, are not known for the individuals partaking in our study. UV-B induced DNA damage delivered at different sun angles, whether the sun is directly above an individual (summer noon) or morning or afternoon where the sun irradiance is lower will impact absorbance (Sloney, 2006). In addition, ground surface reflections play a role in increasing or decreasing the amount of light reaching the eye. Even hazy day conditions can lead to wider corneal opening and may expose the retina to more damage than bright sunlight (Sloney, 2006). Reflective ground surfaces such as snow, where the light is reflected at a high rate would be more damaging than light off a light-absorbing surface like asphalt (Sloney, 2006). This may be why December was a common month for loss of vision in our data set of individuals living in North America (**Fig. 3D, 3E**), though further studies are needed.

This study did find that participant habits surrounding sunglass use nearly doubled after the participant lost their vision (34% to 60%) (**Fig. 4**). However, it is important to note that some studies suggest that sun protective measures such as sun-brimmed hats and sunglasses do not provide sufficient peripheral protection and may not reduce actual UV ocular exposure of critical parts of the lens (Sloney, 2006). Peripheral light may be a risk since traditional sun-shading devices do not block tangential light and in shaded areas the iris of the eye is more relaxed. Therefore, it may be prudent for health care professionals to specifically

recommend “wrap around” sunglasses, which also provide protection to peripheral vision.

Sources of light damage are not limited to sunlight but also bright lights and blue light-emitting-diode (LEDs), which demonstrated more photochemical injury as compared to red and green LEDs (Shang et al. 2017). Similarly, rats exposed to red light at 2,000 Lux exhibited no significant difference in mRNA or protein expression between the control (non-exposed eye) and the exposed eye (Osborne et al. 2008). Yet, rats that received the white light as an insult to one eye had significant differences between the control and exposed eyes (Osborne et al. 2008). Further suggesting that it is the blue/violet region of the spectrum causing damage to the retinal ganglion cells.

This and other studies suggest that mitochondrial oxidative stress should be minimized in asymptomatic LHON carriers (Dimitriadis et al., 2014; Sadun et al., 2003). In this study, participants who experienced vision loss at an older age reported two times the number of exposures than younger participants (**Fig. 5A, 5B**). One possible explanation is that older individuals are more likely to have an accumulation of exposures from activities such as smoking, drinking alcohol, workplace exposures etc. than a younger individual. Another possible explanation is that individuals who lose their vision later in life have genetic modifiers requiring more insult to cause disease onset, as was suggested when studies found that the heaviest smokers had the latest disease onset (Carelli et al., 2015; Yu-Wai-man, Hudson, Klopstock, & Chinnery, 2016). However, an accumulation of environmental exposures is not seen in all individuals with LHON. Nine individuals

from this present study (four under 20 years of age, five over 20 years of age) did not indicate exposure to any of the environmental factors listed in the survey (**Fig. 5**).

Exposures specifically listed by women with LHON were also investigated. There were fifteen total women (32%, **Fig. 1A**) in this study, which is a larger number than expected since only 10% of female carriers usually experience vision loss. Four female participants went through menopause prior to or during vision loss (**Fig. 5**). Two female participants had received hormone replacement therapy. Estrogen has previously been found to positively influence mitochondrial subunit expression (Araújo, Beyer, & Arnold, 2008), which may mitigate reactive oxidative species-induced apoptosis in cultured neuronal cells (Valencia & Morán, 2004). Hormone replacement therapy has been demonstrated to be protective for female carriers of a LHON variant (C. Giordano et al., 2011, Fantini, Asanad, Karanjia, & Sadun, 2018). This effect was noted by one of our participants as well: “I was on estrogen after my hysterectomy. I stopped taking them about 4 months before I started having vision loss. I got back on them 6 months after going blind and within 2 months my eyesight had improved quite a bit.”

The complex etiology of LHON makes it difficult to determine genotype-phenotype correlations and to infer what may modify disease severity, whether it be mitochondrial polymorphisms, nuclear factors, or the duration of environmental exposures at a particular time point during an individual's lifetime. Further prospective research is needed to encapsulate a wide range of possible

environmental and stress-related exposures across a large population of individuals with LHON.

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Conflict of interest

The authors report no conflict of interest.

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Supplemental Tables

Supplemental Table 1. LHON questions on vision loss (A) and exposure evaluation (B).

<p>A. LHON Vision Information: Specific questions relating to your vision.</p> <ol style="list-style-type: none"> 1. What date did you OR your child lose vision in the right eye? Please be as specific as possible with date, month, year. 2. At what AGE, did you OR your child lose vision in the right eye? 3. Have you OR your child had any visual recovery in the right eye? 4. What date did you OR your child lose vision in the left eye? Please be as specific as possible with date, month, year. 5. At what AGE were you OR your child when vision was lost in the left eye? 6. Have you OR your child had any visual recovery in the left eye? 7. How old were you OR your child (in years) when a formal diagnosis of Leber's Hereditary Optic Neuropathy (LHON) was made? 8. Have you OR your child ever had genetic testing? 9. If done, what genetic change was identified? (If unsure, please say unknown) 10. Which family members also are known to have LHON-related vision loss?
<p>B. Possible Exposures: Questions pertaining to your environment.</p> <ol style="list-style-type: none"> 1. At the time of vision loss were you OR your child a smoker? 2. What age did you OR your child start smoking? 3. In the months leading up to your initial vision loss, how often were you OR your child smoking cigarettes? 4. In the months leading up to your initial vision loss, on average, how many cigarettes were you OR your child smoking per week (number on average)? 5. Prior to your initial vision loss, were you OR your child exposed to secondhand smoke on a regular basis (eg. living with a smoker or working at a facility with secondhand smoke)? 6. At the time of your OR your child's initial vision loss were you OR they drinking alcoholic beverages? 7. How old were you OR your child when you OR they had your first alcoholic beverage? 8. In the months leading up to your OR your child's initial vision loss, how many alcoholic beverages were you OR they consuming a week (on average)? 9. Have you OR your child started or completed menopause? 10. What age did you start menopause? 11. During menopause, or during another time point, were you OR your child ever given any hormone replacement therapy (HRT)? 12. Prior to your initial vision loss, were you OR your child ever been exposed to toxic chemicals? 13. How many hours a day did you OR your child spend outside on average as a youth?

14. In the months prior to your OR your child's initial vision loss, how many hours was spent outside?
15. Where were you OR your child living (Province or State, Country) at the time of vision loss?
16. Where you OR your child travelling at the time of initial vision loss?
17. In the months prior to your OR your child's vision loss had you OR your child travelled anywhere?
18. If yes, where did you OR your child travel and for how long?
19. Prior to your OR your child's initial vision loss, did you OR they usually wear sun glasses when outside?
20. Have you OR your child ever been told to protect your OR their eyes from sunlight exposure?
21. Do you OR your child normally wear sun glasses when outside?

Supplemental Table 2. Reported partial recovery of vision loss in our LHON cohort.

	Total (n)	Both eyes	Individual eye	Recovery (%)
m.11778G>A	31	5	2	23
m.3460G>A	6	1	1	33
m.14484T>C	2	1	0	50
m.15512T>?	1	0	0	0
Unknown	7	3	0	43